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Dexamethasone induces the secretion of annexin I in immature lymphoblastic cells by a calcium-dependent mechanism

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Abstract

The mechanisms by which glucocorticoids (GC) regulate annexin I (ANXA1) secretion in different cells are still a matter of debate. The aims of this study were to evaluate the ability of dexamethasone (Dex) to induce ANXA1 secretion and to investigate the roles of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and of the GC receptor, on that process. For this purpose, the human immature lymphoblastic CCRF-CEM cell line was used. Treatment of the cells with Dex, for up to 4 h, significantly reduced the intracellular content of ANXA1 and increased the amount of this protein bound to the outer surface of the plasma membrane, whereas exposure of cells to Dex, for 12 h, induced the synthesis of ANXA1. At the same short time periods, Dex also induced a significant increase in the $[Ca^{2+}]_i$. Incubation of the cells with BAPTA-AM (10 μM), a cell-permeant high affinity Ca^{2+} chelator, completely inhibited Dex-induced ANXA1 secretion. Furthermore, the Ca^{2+} ionophore, ionomycin, alone induced ANXA1 cleavage, but not its secretion. Additionally, we used brefeldin A to investigate the involvement of the classical endoplasmic reticulum (ER)-Golgi pathway of protein secretion in the release of ANXA1. The GC receptor antagonist, RU486, neither reverted the Dex-dependent ANXA1 secretion nor inhibited the increase of the $[Ca^{2+}]_i$ induced by Dex. Together, our results indicate that Dex induces ANXA1 synthesis and secretion in CCRF-CEM cells. ANXA1 secretion in this cell type show the following characteristics: (i) is unlikely to involve the classical ER-Golgi pathway; (ii) requires a Ca^{2+}-dependent cleavage of ANXA1; (iii) involves both Ca^{2+}-dependent and independent mechanisms; and (iv) is apparently independent of the GC receptor alpha isoform. (Mol Cell Biochem **237**: 31–38, 2002)

Key words: lymphoblastic cells, annexin I, dexamethasone, glucocorticoid receptor, secretion, calcium

Introduction

Annexin I (ANXA1) is a well-characterised member of a structurally related family of Ca^{2+}- and phospholipid-binding proteins [1, 2]. This protein has been implicated in cell growth and differentiation [3–5], and in the initiation of membrane fusion and exocytosis [6–8]. ANXA1 also appears to mediate some of the anti-inflammatory actions of glucocorticoids (GCs), including inhibition of chemotaxis, and superoxide, prostaglandin and leukotrien production [9, 10].

Neutrophils and monocytes are particularly enriched in ANXA1 and ANXA1 binding sites, and much attention has been focused on the regulation of ANXA1 expression by GCs in these cell types [11–14]. In contrast, the number of ANXA1 binding sites in lymphocytes is barely detectable, and whether GCs regulate the expression of ANXA1 in these cells is still a matter of debate [15–18]. Moreover, the expression of ANXA1 and its regulation by steroids may also change during the differentiation process [19–21]. Differentiated cells are thought to constitutively express more ANXA1 than the

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undifferentiated cells [3, 22, 23]. Furthermore, the subcellular localisation of ANXA1 seems to depend on the cell type. In different cells, ANXA1 has been detected in the cytosol, bound to the plasma membrane or associated to the cytoskeleton [5, 6, 24].

Several *in vivo* and *in vitro* studies have shown that exposure to endogenous or exogenous GC hormones increases the cellular turnover of ANXA1, changes its subcellular localisation and stimulates the secretion of this protein [3, 11, 16, 22, 24]. However, the mechanisms involved in ANXA1 exportation from the intracellular milieu to the outer surface of the plasma membrane are still not completely understood. ANXA1 does not contain a hydrophobic signal sequence necessary for the release from the intracellular compartment via the classical endoplasmic reticulum (ER)-Golgi system, unlike most proteins that undergo secretion [1, 25]. Some recent studies suggest that its secretion occurs through an unconventional pathway [2, 5, 11, 21], or through a Ca^{2+}-dependent mechanism [6, 7], both yet not totally understood. Once outside the cell, ANXA1 gains access to specific high-affinity, saturable and Ca^{2+}-dependent binding sites [24, 26, 27], which have been clearly identified on the plasma membrane of neuroendocrine and phagocytic cells [3, 15, 16, 18].

The aim of this study was to elucidate whether dexamethasone (Dex) modulates the secretion of ANXA1 in lymphocytes. Furthermore, we also studied the roles of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and of the GC receptor on the Dex-induced ANXA1 secretion. For this purpose we used the human immature lymphoblastic cell line, CCRF-CEM, which we previously showed to express ANXA1 [28]. We found that Dex induced a Ca^{2+}-dependent release of ANXA1, followed by *de novo* synthesis of the protein in the lymphoblastic cells used.

Materials and methods

Culture conditions

The human T cell CCRF-CEM acute lymphoblastic leukemia cell line (ATCC) was maintained in RPMI medium (Sigma Chemical Co.) supplemented with 10% fetal calf serum (FCS) (Biochrom), 100 μg/ml streptomycin and 100 U/ml penicillin (Sigma Chemical Co.), at 37°C, in an atmosphere of 5% CO_2/95% air.

For experiments in which the effect of Dex on ANXA1 expression was determined, the cells were incubated with Dex (Sigma Chemical Co.) (50 nM to 1 μM), for 30 min to 12 h. The steroid antagonist RU486 (Sigma Chemical Co.) (1–10 μM) was used in the presence of Dex (1 μM), to study the involvement of GC receptors.

For experiments in which the measurement of $[Ca^{2+}]_i$ was the endpoint, the cells were incubated with Dex (1 μM), in the presence and in the absence of RU486 (1–10 μM), for 30 min, 2 h or 4 h.

For experiments in which the secretion of ANXA1 was studied, CCRF-CEM cells were incubated with Dex (1 μM) in the presence or in the absence of brefeldin A (Sigma Chemical Co.) (1 μM to 1 mM), for 4 h. To determine the effect of the $[Ca^{2+}]_i$ on ANXA1 secretion, CCRF-CEM cells were pretreated with BAPTA-AM (1,2-bis (o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester) (Molecular Probes) (10 μM), 30 min before the incubation with Dex (1 μM), or with the Ca^{2+} ionophore, ionomycin (Molecular Probes) (3 μM), for 5–10 min.

All these experiments were performed with cells plated (1×10^6 cells/ml) in culture dishes and pre-incubated, for 14 h, with RPMI supplemented with 2.5% FCS and antibiotics. When CCRF-CEM cells were maintained in 10% FCS-supplemented RPMI, they expressed a very high basal level of ANXA1 that masked the effect exerted by Dex on protein secretion (data not shown). In each experiment, controls were always included by incubating the cells in the corresponding volume of culture medium plus the appropriate vehicle.

Viability of the CCRF-CEM cells was always checked by the trypan blue exclusion and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Co.) reduction test prior to every experiment performed. Assessment of MTT reduction by metabolically active cells was made by a colorimetric assay, as previously described [29, 30].

Western blot analysis

The secreted plasma membrane-bound ANXA1 was extracted with 100 μl of phosphate buffer saline (PBS) containing 1 mM ethylenediaminotetraacetic acid (EDTA), as previously described [2, 31]. Cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.6, 10% glycerol, 5 mM β-mercaptoethanol), and a protease inhibitor cocktail (Roche) and then sonicated. Samples of cellular lysates (30 μg) and secreted proteins (2 μg) were added (1:1) to denaturating buffer (200 mM Tris, 200 mM bicine, 8 M urea, 4% SDS, 10% β-mercaptoethanol and 0.2% bromophenol blue), boiled, subjected to 12% SDS-PAGE, and electrotransferred to a PVDF (polyvinylidene difluoride) membrane. The membrane was then blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) with 0.1% Tween 20. ANXA1 was detected by incubation with a mouse anti-ANXA1 monoclonal antibody (BabCO) (2 μg/ml), followed by incubation with a horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce). The immunocomplexes were visualised by the ECL chemiluminescent method. To demonstrate equivalent total protein loading, the membranes were stripped with stripping buffer (100 mM β-mercaptoethanol,

2% SDS, 62.5 mM Tris-HCl, pH 6.7), at 55°C, for 45 min. After washing with TBS with 0.1% Tween 20, the membrane was treated as indicated above, using the anti-actin mouse monoclonal antibody (Roche) (0.1 µg/ml). The Ponceau S and Indian Ink staining of the PVDF membrane was used to show equivalent secreted protein loading.

Measurement of intracellular free Ca^{2+} concentration

In the final 30 min of each incubation with Dex, or RU486 plus Dex, the cells were loaded with fura-2/AM (Molecular Probes) (3 µM). The cells were then rapidly washed by centrifugation and resuspended in a Na^+-salt solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose, 10 mM Hepes). The Ca^{2+} measurements were performed with 2 ml of cell suspension (1×10^6 cells) in a Perkin Elmer LS-5B luminescence spectrometer, at a constant temperature of 37°C. Excitation wavelength was 340 nm (5 nm slit), and the emission wavelength was 510 nm (10 nm slit). The $[Ca^{2+}]_i$ corresponding to fluorescence emitted by trapped fura-2 was calculated by the equation: $[Ca^{2+}]_i = 224\ nM \times (F–F_{min})/(F_{max}–F)$; $F_{min} = AF + (F_{max}–AF)/3$ (where 224 nM is the K_d value for fura-2). F_{max} and AF (autofluorescence) were obtained after the addition of ionomycin (3 µM) and $MnCl_2$ (2.25 mM), respectively, as previously described [32].

Statistical analysis

Results were expressed as means ± S.D. Comparison of data from 2 treatment groups was made by the Student's two-tailed unpaired *t*-test. Comparison of data from more than 2 treatment groups was made by the one-way ANOVA analysis with Dunnett's post test. A probability of less than 5% (i.e. $p < 0.05$) was taken as statistically significant.

Results

Effect of Dex on the intra and extracellular levels of ANXA1

In order to test whether Dex influences the cellular content of ANXA1 in the CCRF-CEM cell line, the cells were incubated in the absence (control) or in the presence of different concentrations of Dex (from 50 nM to 1 µM). Although Dex appeared to influence the cellular ANXA1 content at 100 nM, a significant, measurable and reproducible effect was only observed with 1 µM Dex. Therefore, 1 µM Dex was the concentration used in all the experiments performed in this work.

The ANXA1 externally bound to the plasma membrane can be removed by washing the cells with a Ca^{2+}-free salt solution, containing 1 mM EDTA, which by chelating Ca^{2+} releases the extracellular ANXA1 into the medium, as previously described [2, 31]. Therefore, the secreted ANXA1 was recovered in the Ca^{2+}-free salt solution used to wash the cells, and the corresponding cellular lysates were used for the detection of intracellular ANXA1.

The Western blot analysis presented in Fig. 1 show that treatment of the cells with Dex (1 µM), for 30 min or 4 h, decreased the level of intracellular ANXA1 to 50.7 ± 2.7 % and 48.4 ± 5.7%, respectively, of that found in control untreated cells ($p < 0.01$). The intracellular content of ANXA1 was restored, back to control levels (105.4 ± 2.1% relatively to the control), by treatment of the cells with Dex, for 12 h.

The loss of intracellular ANXA1 is unlikely to be caused by leakage of the protein from dead or damaged cells, as the viability of the cells treated with Dex, for 30 min, 2, 4 or 12 h, was always > 95% relatively to control cells. The cell viability was determined by the trypan blue exclusion test and by the MTT assay (data not shown). Therefore, the decrease in the cellular ANXA1 content, as shown in Fig. 1A, is likely to be due to a specific secretion of the intracellular protein.

The EDTA-extractable plasma membrane-bound ANXA1 defines the total amount of ANXA1 attached to the external

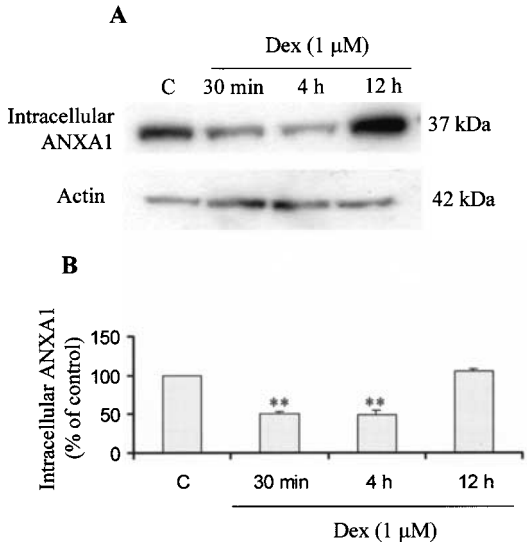

Fig. 1. Effect of Dex on the intracellular level of ANXA1. CCRF-CEM cells were incubated in the absence (control, C) or presence of Dex (1 µM), for 30 min, 4 h or 12 h. ANXA1 externally bound to plasma membranes was removed with an EDTA solution, as described in 'Materials and methods'. The intracellular ANXA1 content was determined by Western blot analysis, using a mouse monoclonal anti-ANXA1 antibody (A), and the bands were quantified with an image analyser (B). The data shown are an average of four independent experiments. The values are means ± S.D., where **$p < 0.01$, as determined by one-way ANOVA with Dunnett's post test.

surface of the plasma membrane, and indirectly reflects the amount of secreted ANXA1 [2, 31]. The results presented in Fig. 2 indicate that stimulation of CCRF-CEM cells with Dex (1 μM) increased the amount of plasma membrane-bound ANXA1, reaching statistical significance after stimulation with Dex for 4 and 12 h (206.1 ± 13.0 and 255.5 ± 18.0% of control, respectively) ($p < 0.01$). The secreted ANXA1 appeared as a doublet of bands with 37 and 34 kDa (Fig. 2A).

The steroid antagonist, RU486, was used in order to determine whether the effect of Dex on ANXA1 secretion could be attributed to activation of GC receptors. RU486 (1–10 μM) neither affected the amount of plasma membrane-bound ANXA1 in the absence of Dex, nor did it revert, at any of the concentrations used, the Dex-dependent secretion of ANXA1 in CCRF-CEM cells (data not shown).

To elucidate the mechanisms by which Dex stimulates the secretion of ANXA1, we used brefeldin A (Bref A), which blocks the classical endoplasmic reticulum-Golgi pathway of protein secretion. For this purpose, CCRF-CEM cells were treated with Dex (1 μM), for 4 h, in the absence or presence of Bref A. The results presented in Fig. 3 show that Bref A, in concentrations ranging from 1 μM to 1 mM, was unable to inhibit the effect of Dex on the intracellular content of ANXA1. However, in the presence of 1 mM Bref A the observed effect of Dex was reverted, although incompletely.

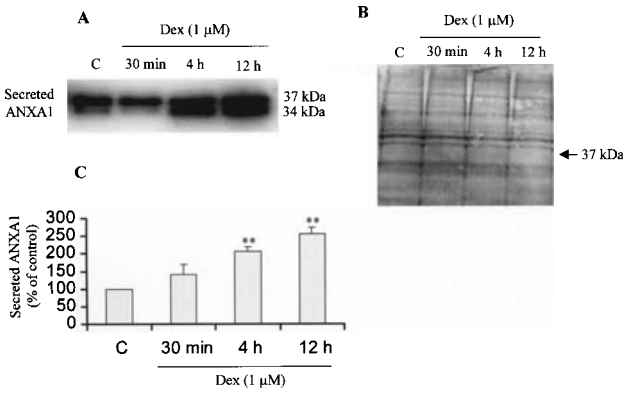

Fig. 2. Effect of Dex on the secretion of ANXA1. CCRF-CEM cells were incubated in the absence (control, C) or presence of Dex (1 μM), for 30 min, 4 h or 12 h. Secreted plasma membrane-bound ANXA1 was extracted by washing the cells with a Ca^{2+}-free salt solution, containing 1 mM EDTA, as described in 'Materials and methods'. The secreted ANXA1 level was determined by Western blot analysis, using a mouse monoclonal anti-ANXA1 antibody (A), the PVDF membrane was stained with Indian Ink (B), and both bands in each lane were quantified as one with an image analyser (C). The data shown are an average of 4 independent experiments. The values are means ± S.D., where $^{**}p < 0.01$ as determined by one-way ANOVA with Dunnett's post test.

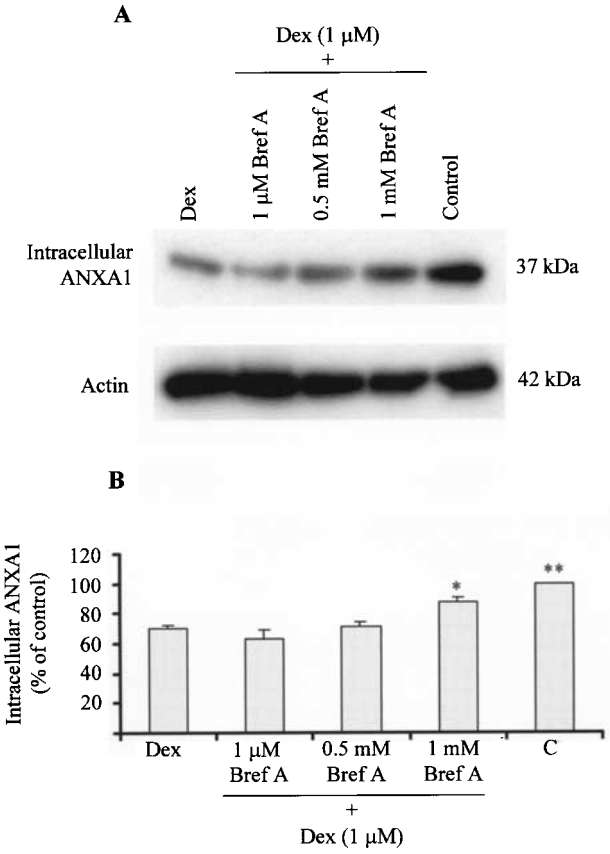

Fig. 3. Effect of Bref A on the secretion of ANXA1. CCRF-CEM cells were incubated without (control), or with Dex (1 μM) in the absence or presence of Bref A (1 μM to 1 mM), for 4 h. ANXA1 externally bound to plasma membrane was removed with an EDTA solution, as described in 'Materials and methods'. The intracellular ANXA1 content was determined by Western blot analysis, using a mouse monoclonal anti-ANXA1 antibody (A), and the bands were quantified with an image analyser (B). The data shown are an average of 3 independent experiments. The values are means ± S.D., where $^{**}p < 0.01$ and $^{*}p < 0.05$, as determined by one-way ANOVA with Dunnett's post test.

Effect of Dex on the $[Ca^{2+}]_i$

In order to evaluate whether Dex affects the $[Ca^{2+}]_i$ in the CCRF-CEM cell line, the cells were incubated in the absence (control) or presence of Dex (1 μM), and the $[Ca^{2+}]_i$ was assessed using the fluorescent indicator fura-2. As shown in Fig. 4, the level of $[Ca^{2+}]_i$ in control cells was 175.9 ± 13.0 nM, slightly increasing, after incubation with Dex (1 μM) for 30 min, to 221.4 ± 16.1 nM. The $[Ca^{2+}]_i$ increased further when the cells were exposed to Dex (1 μM) for 2 h (236.6 ± 16.1 nM) or 4 h (210.1 ± 21.8 nM) ($p < 0.05$).

The steroid antagonist, RU486, was used to investigate the putative role of GC receptors in the Dex-dependent $[Ca^{2+}]_i$ rise. CCRF-CEM cells were incubated in the absence (control) or presence of RU486 (10 μM) plus Dex (1 μM). The rise in

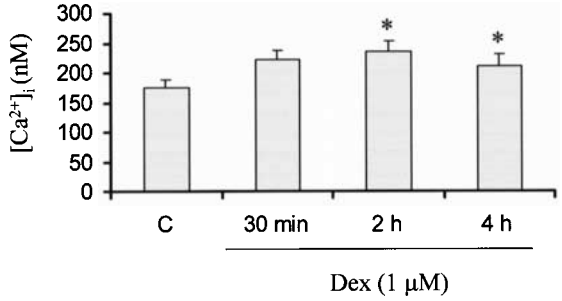

Fig. 4. Effect of Dex on the $[Ca^{2+}]_i$. CCRF-CEM cells were incubated in the absence (control, C) or in the presence of Dex (1 μM), for 30 min, 2 h and 4 h. The $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator fura-2, and the $[Ca^{2+}]_i$ was calculated as described in 'Materials and methods'. The data shown are an average of 4 independent experiments. The values are means ± S.D., where *$p < 0.05$ as determined by one-way ANOVA with Dunnett's post test.

the $[Ca^{2+}]_i$ evoked by stimulation of the cells with Dex (1 μM), in the presence of RU486 (10 μM), was not significantly different from that obtained in cells treated with Dex alone (data not shown).

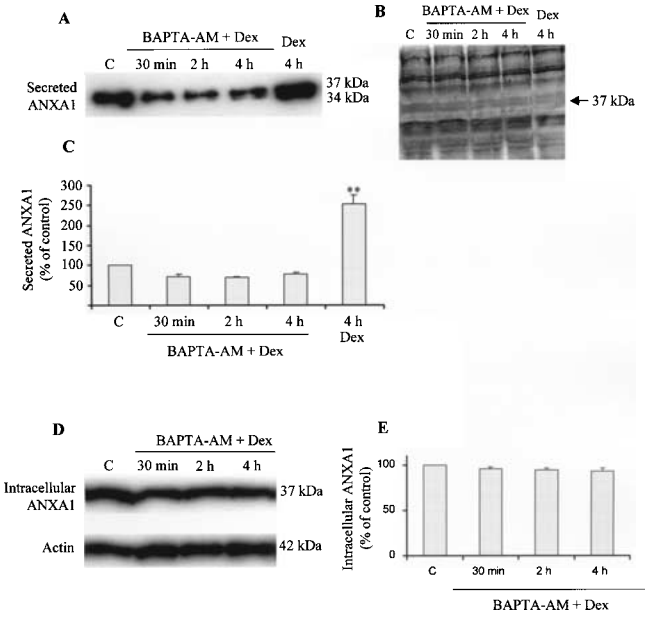

Fig. 5. Effect of the $[Ca^{2+}]_i$ on the secretion of ANXA1. CCRF-CEM cells were incubated without (control, C), or with Dex (1 μM) in the absence or presence of BAPTA-AM (10 μM), for 30 min, 2 h and 4 h. ANXA1 externally bound to plasma membranes was removed by washing the cells with a Ca^{2+}-free salt solution, containing 1 mM EDTA, as described in 'Materials and methods'. The secreted and the intracellular ANXA1 were determined by Western blot analysis using a mouse monoclonal anti-ANXA1 antibody (A, D), the PVDF membrane was stained with Indian Ink (B), and the bands were quantified with an image analyser (C, E). The data shown are an average of 3 independent experiments. The values are means ± S.D., where **$p < 0.01$ as determined by one-way ANOVA with Dunnett's post test.

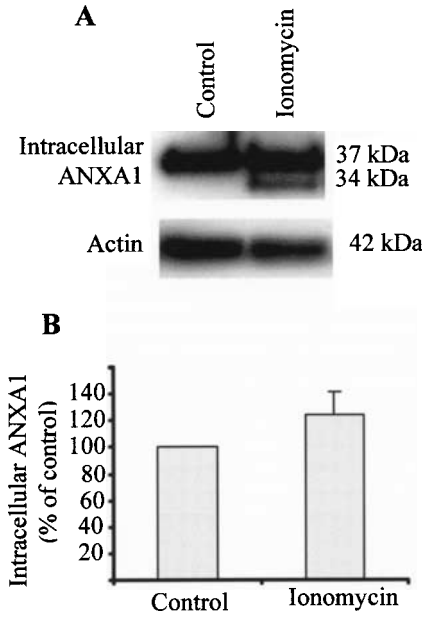

Fig. 6. Effect of Ionomycin on the intracellular level of ANXA1. CCRF-CEM cells were incubated in the absence (control) or presence of ionomycin A (3 μM), for 10 min. ANXA1 externally bound to plasma membranes was removed with an EDTA solution, as described in 'Materials and methods'. The intracellular ANXA1 content was determined by Western blot analysis, using a mouse monoclonal anti-ANXA1 antibody (A), and the bands were quantified with an image analyser (B).

Effect of the $[Ca^{2+}]_i$ on ANXA1 secretion

Finally, we studied whether the effects of Dex on the secretion of ANXA1 could be attributed to the increase in $[Ca^{2+}]_i$ induced by this steroid. For this purpose, CCRF-CEM cells were pre-loaded with the Ca^{2+} chelator BAPTA-AM, before stimulation with Dex.

Treatment of the cells with Dex (1 μM), for 4 h, induced an evident increase in the secreted ANXA1 (253.3 ± 21.8% relatively to control) ($p < 0.01$), whereas, in the presence of BAPTA, the amount of cell surface ANXA1 was dramatically reduced, reaching levels equivalent to those found in control untreated cells (Figs 5A and 5C). Additionally, analysis of the corresponding intracellular extracts from cells pre-treated with BAPTA-AM (10 μM) did not show differences relatively to the control (Figs 5D and 5E).

To further elucidate the role of the $[Ca^{2+}]_i$ on ANXA1 secretion induced by Dex, we used the Ca^{2+} ionophore ionomycin. The results in Fig. 6 show that ionomycin (3 μM) by itself induced no significant change in the level of intracellular ANXA1, but it induced the appearance of both the 34 and 37 kDa ANXA1 bands which, after stimulation with Dex, were detectable only in the extracellular fraction.

Discussion

Differentiated cells are thought to constitutively express more ANXA1 than undifferentiated cells [3, 21, 22]. However mature lymphocytes express low amounts of ANXA1 and of ANXA1 binding sites [15, 23, 33], and in our previous work we observed that the immature lymphoblastic CCRF-CEM cells express a considerable amount of intracellular ANXA1 [28]. In this work we found that Dex induces the secretion of ANXA1 in this lymphoblastic cells by a Ca^{2+}-dependent mechanism.

In the absence of Dex, the bulk of ANXA1 was found inside the CCRF-CEM cells, and a small proportion of ANXA1 was associated with the outer surface of the plasma membrane (Figs 1 and 2). Short periods (up to 4 h) of stimulation with Dex caused a pronounced, and time-dependent, increase in the plasma membrane-bound ANXA1 (Fig. 2), while the intracellular ANXA1 level (non-extractable with Ca^{2+}-free medium) decreased to approximately 50% of that found in control untreated cells (Fig. 1).

Expression of ANXA1 has been described as a process dependent on the stage of cell differentiation. For example, Dex increased ANXA1 synthesis and secretion only when U-937 cells (an immature monocytic cell line) were differentiated by treatment with phorbol 12-myristate 13-acetate [21, 34]. However, the results of this work show that the immature CCRF-CEM cells possess a Dex-dependent mechanism of ANXA1 secretion, which is in accordance with most of the previous studies in mature cells, including human neutrophils and monocytes [11, 13, 35].

In several cell types, the reduction of the intracellular pool of ANXA1, in response to Dex, is accompanied by *de novo* synthesis of the protein [3, 24]. We found that stimulation with Dex, for 12 h, induced a replenishment of the intracellular content of ANXA1 (Fig. 1), and a significant increase in the released protein (Fig. 2), indicating that in the lymphoblastic CCRF-CEM cell line Dex also upregulates ANXA1 synthesis.

Little is known about the mechanism by which ANXA1 is secreted in response to GCs. ANXA1 has been reported to lack the common hydrophobic signal sequence required for the release via the ER-Golgi system [11]. Although, some evidences indicate that ANXA1 secretion occurs through a mechanism which culminates with exocytosis [2], others found opposing evidence [5]. Since the classical ER-Golgi pathway appears not to be involved in ANXA1 secretion [2, 11], an unknown alternative pathway is generally admitted.

Bref A reversibly blocks the anterograde transport of proteins from the ER to the Golgi apparatus [2]. In contrast to previous reports [2, 11], our results show that Bref A inhibited the secretion of ANXA1 in response to Dex (Fig. 3). However, those studies used micromolar concentrations of Bref A [2, 11], whereas the inhibitory effect, observed in this work, was only achieved with concentrations in the millimolar range.

In different cells, micromolar concentrations of Bref A blocked the classical ER-Golgi pathway preventing the secretion of some proteins, but not ANXA1 [2]. Since micromolar concentrations of Bref A are sufficient to inhibit classical pathway of protein secretion, it is not likely that inhibition of ANXA1 secretion occurred as a consequence of the blockade of that pathway. Instead, it is more likely that Bref A blocked another pathway of protein secretion by a non-specific mechanism, which, consequently, required higher concentrations of this compound. On the other hand, Bref A did not completely inhibit Dex-induced ANXA1 secretion. This may suggest that Dex-induced ANXA1 secretion involves another pathway that is not sensitive to Bref A. Nevertheless, the lack of complete blockade of ANXA1 secretion may solely reflect the involvement of a non-specific action of Bref A. Taken together, our results suggest that the classical pathway of protein secretion is not likely involved in Dex-induced ANXA1 secretion in the lymphoblastic CCRF-CEM cells, which is in agreement with studies in other cells [2, 11, 34]. Furthermore, our results also suggest that Dex-induced ANXA1 secretion involve one or more , yet unidentified, secretory pathways which require high concentrations of Bref A to be inhibited.

One possibility is that the process of secretion of ANXA1 is similar to that described for interleukin-1β, which involves the proteolytic cleavage of a N-terminal peptide [2, 11, 21]. Indeed, our results show that following stimulation with Dex, a 34 kDa species of ANXA1 is present in the extracellular (secreted) fraction, together with the native 37 kDa form of ANXA1. The appearance of both species of ANXA1 (34 and 37 kDa) in the extracellular medium has already been described [31] and ANXA1 cleavage has been considered a preliminary step for the secretion process [7, 25, 35]. Accordingly, we found that only the full length ANXA1 (37 kDa) is present in the intracellular fraction from Dex-treated cells (Fig. 1), suggesting that all the cleaved protein was secreted.

Studies in neutrophils [6, 7] and HL-60 cells [36] suggest that the secretion of ANXA1 may be attributed to an increase in the $[Ca^{2+}]_i$. Furthermore, in monocytic cells, Dex has been shown to increase the $[Ca^{2+}]_i$. This suggests that, in some cells, Ca^{2+} signalling pathways may be involved in mediating the cellular effects of Dex [37]. Accordingly, we found that Dex caused a significant increase in the $[Ca^{2+}]_i$ in the lymphoblastic CCRF-CEM cells (Fig. 4). In order to investigate the role of the Dex-induced increases in the $[Ca^{2+}]_i$ in ANXA1 secretion, we used the fast high affinity Ca^{2+} chelator, BAPTA. The cell-permeant BAPTA-AM is converted by intracellular esterases into BAPTA, which selectively buffers $[Ca^{2+}]_i$ changes and depletes stores by direct Ca^{2+} binding [38]. We found that the Dex-induced ANXA1 secretion was completely reverted by pre-treatment of the cells with BAPTA-

AM (Figs 5A and 5C), indicating that ANXA1 secretion requires a rise of the $[Ca^{2+}]_i$. Furthermore, the results on Fig. 5 D show that only the full length ANXA1 (37 kDa) is present in the intracellular fraction of cells treated with BAPTA-AM and Dex.

In order to further elucidate the role of the $[Ca^{2+}]_i$ on the ANXA1 secretion, we used the Ca^{2+} ionophore, ionomycin (Fig. 6). The results obtained show that ionomycin did not reduce the total intracellular content of the protein. Nevertheless, the elevation of the $[Ca^{2+}]_i$ induced by ionomycin, was sufficient for the cleavage of ANXA1 to occur, since both the 34 and 37 kDa species were found in the intracellular fraction (Fig. 6 A). These results indicate that, in the lymphoblastic cells used, the cleavage of ANXA1 is a Ca^{2+}-dependent mechanism, which is in accordance with previous studies in neutrophils [7].

Taken together, the results obtained with the Ca^{2+} chelator BAPTA and with the Ca^{2+} ionophore ionomycin, suggest that ANXA1 cleavage is a Ca^{2+}-dependent process, which is essential for and precedes ANXA1 secretion. Nevertheless, although required for the cleavage of ANXA1, the increased $[Ca^{2+}]_i$ is not sufficient for the secretion of the protein, since ionomycin alone did not decrease the intracellular levels of ANXA1 (Fig. 6). Therefore, besides this Ca^{2+}-dependent signalling pathway, other intracellular processes have to be activated by Dex, in lymphoblastic cells, so that ANXA1 secretion can be accomplished.

The effects of Dex on ANXA1 secretion and on changes of the $[Ca^{2+}]_i$ in different cells have been attributed to the activation of GC receptors [2, 22, 39, 40]. Several evidences show that RU486, a potent GC receptor antagonist, has been commonly used to study the involvement of GC receptors on the expression of annexin 1 induced by GCs [1, 3, 22, 39]. Therefore, in this work we used RU486 to investigate whether the effects of Dex reported here were mediated by the GC receptor. Although CCRF-CEM cells constitutively express the intracellular GC receptor alpha isoform, RU486 (10 μM), failed to inhibit the effect of Dex on the $[Ca^{2+}]_i$ and on ANXA1 secretion (data not shown), suggesting that these receptors unlikely mediate the effects of Dex reported here. On the other hand, these effects of Dex were only consistent and significant when high concentrations (1 μM) of Dex were used. This also argues against the involvement of the GC receptor, since ligand-receptor interactions usually occur with much lower concentrations. However, we observed that concentrations of RU486 higher than 10 μM were toxic to the cells (viability < 70% relatively to control cells) (data not shown), which limited the possibility of further elucidating the involvement of the GC receptor in the responses induced by Dex. Therefore, we cannot definitely exclude the involvement of the intracellular GC receptor alpha isoform in ANXA1 secretion. However, other studies showed that the effects of Dex may occur through RU486-insensitive GC plasma membrane binding sites [40] that have been previously described in CCRF-CEM cells [41], and/or via non-genomic mechanisms, such as the mitogen-activated protein kinases [42] or protein kinase C [43, 44].

In summary, our results indicate that Dex induces the synthesis and secretion of ANXA1 in the human immature lymphoblastic CCRF-CEM cells, and that Dex-induced ANXA1 secretion results from the combination of several processes, each of which is essential, but not sufficient, for ANXA1 secretion to be accomplished.

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